easily removed by sedimentation. The δ-disilicate, though insoluble during the wash process, dissolves when the solution becomes dilute during the rinse cycle.

Future Trends

The builder development for detergents is now more and more driven by environmental issues. Zeolite A was a major breakthrough over the conventional phosphates. An ideal builder would be one combining the efficacy of STPP and the environment friendly nature of (alumino) silicates. At this point, combination of builders and co-builders is the way detergents are being formulated. The new materials zeolite MAP and δ-disilicate, are the future; though more efficient, they still have to cross the cost barrier to become major players in the detergent industry. As efforts are constantly underway to come up with more and more efficient builder and detergent formulations, the challenge is to achieve compatibility, environment friendliness, and low production cost, all at the same time.

See also: **II/Ion Exchange:** Inorganic Ion Exchangers; Novel Layered Materials: Phosphates; Novel Layered Materials: Non-Phosphates.

Further Reading

Adams CJ, Araya A, Carr SW *et al.* (1997) Zeolite MAP: The new detergent zeolite. *Studies in Surface Science and Catalysis* 105: 1667.

Breck DW (1974) *Zeolite Molecular Sieves: Structure, Chemistry and Use*. New York: John Wiley.

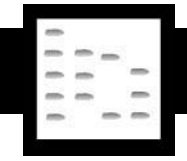
Cutler WG and Kissa E (eds) (1987) *Detergency Theory and Technology*. New York: Marcel Dekker.

Showell MS (ed.) (1998) *Powdered Detergents*. New York: Marcel Dekker.

DNA

See **III / DEOXYRIBONUCLEIC ACID PROFILING: Overview; Capillary Electrophoresis**

DRUGS AND METABOLITES



DropBooks

Liquid Chromatography–Mass Spectrometry

R. P. W. Scott, Avon, CT, USA

Copyright © 2000 Academic Press

The tandem analytical instrument, comprising a liquid chromatograph and a mass spectrometer is ideally suited for identifying drug metabolites and for following their metabolic pathways. The segregation of each drug metabolite from the sample matrix, and its subsequent identification, firstly requires a very efficient separation technique. As the metabolites are often chemically very similar to the parent drug, this exigency is adroitly furnished by the liquid chromatography (LC) column (usually microbore) packed with very small particles. Secondly, the materials of interest are inevitably present in the biological system in very small quantities and thus, despite the use of sample concentrating techniques, a very sensitive detection technique is essential. The required high sensitivity is effectively supplied by the ion multiplier of the mass spectrometer sensor. Thirdly, in order to identify the individual metabolites, structural information is required which must be highly specific and contain adequate detail at high resolution. Such data is readily provided by the mass spectrum of each eluted solute, which can either be compared with standard spectra or identified by using well-established interpretation procedures. In some cases, the liquid chromatograph can be coupled to a MS/MS instrument that will provide mass spectra of each fragment ion, from each eluted solute, should more detailed information be needed.

For the effective use of LC/MS, special sample preparation techniques are necessary and suitable LC/MS interfaces should be employed. Both these subjects are discussed in detail in other parts of the Encyclopedia, and it will be sufficient here to give the sample preparation details of each typical application that is discussed. In addition, the general interface systems that are used, such as thermospray, electrospray, atmospheric pressure chemical ionization devices, transport interfaces etc., are also described in

detail under LC interface devices and can be referred to when appropriate. Drug and drug metabolites find their way to many parts of the body in a variety of ways: via the alimentary canal, via the blood stream, or through the lymph system, and sometimes by direct diffusion through specific tissues. In animal tests, drug and drug metabolites can be found, extracted and assayed in many specialized tissues such as the liver, kidneys, heart muscle, brain, etc. and are usually extracted using standard procedures after homogenizing the tissue. In human tests, the drug and drug metabolites are usually measured in biological fluids, such as whole blood, blood plasma, gastric fluids, urine, saliva, etc., although occasionally samples of tissue from biopsies are also examined. In simple cases of drug monitoring, the concentration of a particular drug and its metabolites may be monitored during treatment merely to ensure that the drug level is kept at an optimum for prime response. In more complicated procedures, the drug and its metabolites may be monitored in a number of different fluids and tissues to determine not only the metabolic pathway, but also those tissues and sites where specific types of metabolism or drug breakdown occur.

The unique advantages of the various techniques and procedures used in LC/MS for monitoring drugs and their metabolites are best illustrated by means of a number of carefully selected applications. There is a wide range of LC/MS applications available for drug and drug metabolite analysis and the following have been chosen as typical examples of the use of the technique in pharmacology. Furazolidone (*N*-(5-nitro-3-furfurylidene-3-amino)-2-oxazolodinone) is a 5-nitrofuran antibiotic that is added to animal feeds to help prevent such infections as *Escherichia coil* and *Salmonella* in cattle, pigs and poultry. It follows that it would be important to know the amount of residues (if any) remaining after slaughter in any meat used for human consumption. In Europe, for example, the maximum amount of furazolidone that is tolerated is 5 μg kg^{-1} of animal foodstuff, Furazolidone is light sensitive and so operations must be carried out under artificial yellow light. An example of the measurement of furazolidone in some pharmacokinetic studies is afforded by the work of McCracken *et al*. who used thermospray ionization LC/MS for its determination. The procedure that was used is as follows. 2-g samples of liver and muscle tissue were homogenized with 40 mL of a mixture of McLlvaine buffer and methanol (7:3) and then centrifuged for 15 min. The supernatant liquid was then removed and evaporated down to 15 mL at 40°C. 25 mL of dichloromethane was then added and the mixture shaken for about 1 min. The upper aqueous layer was discarded and the solvent extract carefully evaporated to dryness and then dissolved in a mixture of 2 mL of dichloromethane and 6 mL of hexane. The solution was then extracted by passing it through a prepared Bond-Elut NH_2 extraction cartridge, which was then washed with 5 mL of a hexane/dichloromethane mixture (1:1) and 2 mL of a hexane/chloroform mixture (1:1). The cartridge was then extracted with 5 mL of a mixture of chloroform and methanol (7:3) and the extract evaporated to dryness. The residue was then redissolved in 100 μL of mobile phase, which consisted of 0.1 mol L^{-1} ammonium acetate/acetonitrile (3:1). The sample was injected onto a reversed phase column, 12.5 cm long and 4 mm i.d., containing RP18 stationary phase bonded to 5-μm particles. Chromatograms showing the elution of the Furazolidone by single ion monitoring are shown in **Figure 1**. It is seen that as a result of the use of single ion monitoring, the procedure can be made highly selective. The recovery of the drug ranged between 65 and 70%. The minimum detectable level of contamination was about 1 μg kg^{-1} of tissue, which was quite adequate to confirm that a sample did not contain the drug in excess of the tolerance level.

Another example of the use of the thermospray interface for monitoring drugs in cellular matter from animals is given in the work of Cannavan *et al*. who developed a technique for measuring levamisole in pig and sheep tissue. Levamisole L-(−)-2,3,5,6-tetrahydro-6-phenylimidazole(1,1-b)thiazole, the laevorotatory isomer of tetramisole, is an anthelmintic drug used to control gastrointestinal parasites in cattle, pigs and sheep. In a similar way to that used in the assay of furazolidone, a somewhat complicated sample preparation procedure was necessary. 3 g of the tissue sample was mixed with 2 g of anhydrous sodium sulfate, 9 mL of ethyl acetate and 0.5 mL of 50% (w/v) potassium hydroxide solution, and the mixture homogenized. The mixture was then centrifuged and 6 mL of *n*-hexane was added to 6 mL of the supernatant liquid, and then passed through a Baker Bond CN cartridge column. The column was washed with 5 mL of chloroform/*n*-hexane mixture (1:1) and air-dried. The levamisole was eluted with two 5-mL aliquots of methanol, evaporated to dryness, and the residue taken up in 200 μL of the mobile phase consisting of acetonitrile–tetrahydrofuran–triethylamine–water (350:50:2:598 v/v) containing ammonium acetate (0.1 mol L^{-1}). This solution was then used for the LC/MS analysis. 50-μL samples were placed on a Li-Chrospher 60RP-select B column (12.5 cm long, 4 mm i.d), packed with 5-μm particles. Then mass spectrometer was the Hewlett-Packard 5989A MS engine with thermospray. Single ion

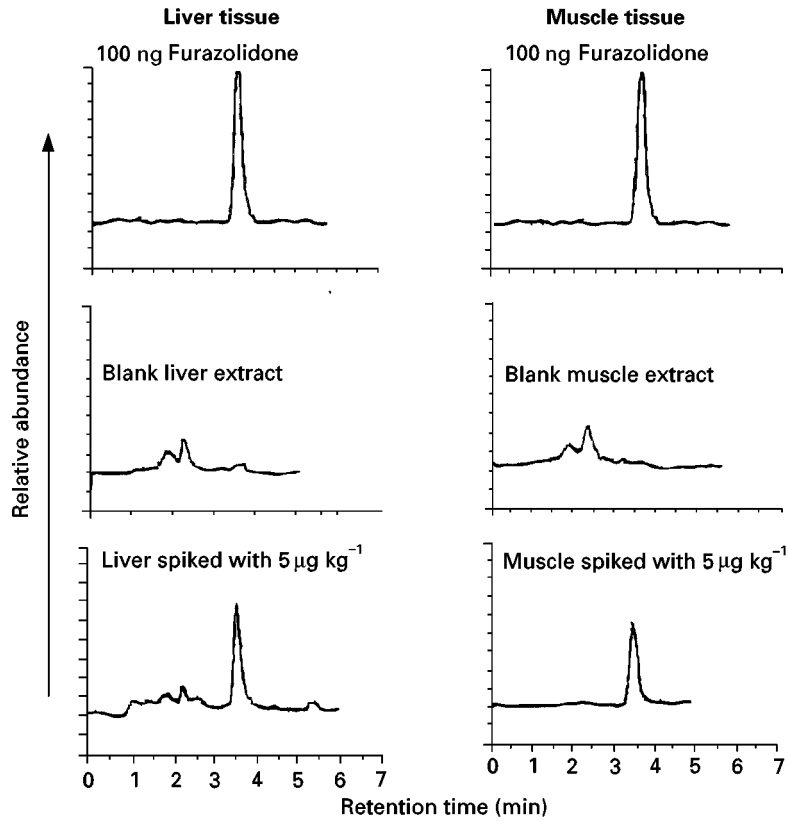

Liver tissue
100 ng Furazolidone
Muscle tissue
100 ng Furazolidone
Relative abundance
Blank liver extract
Blank muscle extract
Liver spiked with 5 μg kg−1
Muscle spiked with 5 μg kg−1
0 1 2 3 4 5 6 7
0 1 2 3 4 5 6 7
Retention time (min)

Figure 1 Single ion chromatograms ($m/z = 243$) of furazolidone demonstrating the sensitivity levels obtainable. (Reproduced with permission from McCracken *et al.*, 1995.)

DropBooks

chromatograms obtained from the analysis are shown in **Figure 2**. They again emphasize the advantages of single ion monitoring; the peak of interest is selected from a complex mixture of peaks with only one other appearing in the chromatogram. The limit of detection was found to be about 5 ng g^{-1} of tissue and the calculated mean recovery for liver, kidney and muscle tissue was found to be 93, 85 and 79%, respectively.

Many penicillins, particularly penicillin G, are extensively used in veterinary medicine. It follows that to prevent antibiotic residues from entering the human food chain, their levels in animal products that have been prepared for human consumption need to be carefully monitored. Blanchflower *et al.* developed a procedure for simultaneously monitoring five penicillins, including oxacillin, cloxacillin and dicloxicillin in both muscle and kidney tissue and also in milk. Blanchflower *et al.* employed an LC/MS tandem instrument fitted with an electrospray interface, and utilized single ion monitoring to selectively locate and measure each antibiotic. The extraction process was exceedingly complicated. An appropriate tissue sample was pulverized, spiked with an appropriate standard and homogenized. Acetonitrile was then added to the homogenate, which was sonicated and finally centrifuged. A portion of the supernatant liquid was then treated with phosphoric acid mixed with dichloromethane and again centrifuged. Acetonitrile and *n*-hexane were added, the mixture shaken and again centrifuged. The lower layer was separated and then washed with water. The solvent mixture was then extracted with phosphate buffer, centrifuged once again and the lower layer treated with tetrabutylammonium hydrogen sulfate. The solution was then extracted with dichloromethane, the extract evaporated to dryness and dissolved in an acetonitrile water mixture. Separations were performed on an Intersil ODS-2 reversed-phase column (15 cm long and 4.6 mm i.d). The outlet from the column was coupled to a Megabore probe of a VG Platform ES-MS, which was operated in the negative ion mode. The source was maintained at 120°C and the flow rate of the drying and nebulizing gas was 10 L h^{-1}. The extraction and focus voltages were about 17 and 24 V, respectively. It was found that the fragmentation pattern was significantly changed by adjusting the voltage on the extraction cone. As the voltage was increased, the degree of fragmentation increased. The effect of extraction cone voltage is

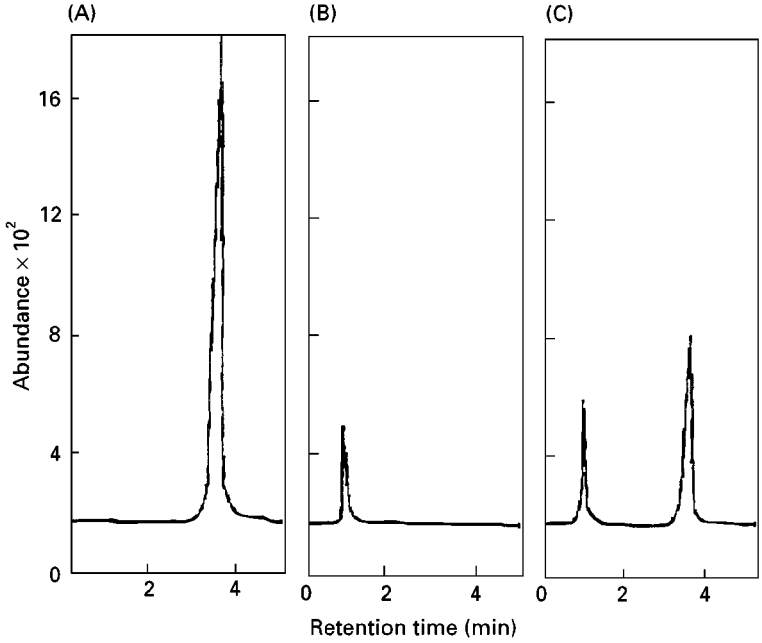


Figure 2 Single Ion chromatograms of levamisole extracted from liver tissue. (A) Levamisole standard (1 μm mL^{-1}), (B) negative liver sample, and (C) liver samples + levamisole (34.8 ng g^{-1}). (Reproduced with permission from Cannavan *et al.*, 1995.)

demonstrated in **Figure 3**. It would appear that a potential of 5 V on the extraction cone produced just two ions above an m/z value of 160, i.e. $m/z = 434$ and 436. Increasing the potential to 10 V produced another peak at an m/z value of 293. At a potential of 20 V, the peak at an m/z value of 293 has markedly increased and has been joined by a significant peak at $m/z = 295$. At the same time, the original major peak at $m/z = 434$ had shrunk to a minor peak and peak at $m/z = 436$ was barely visible. It is obvious that the operating conditions of the interface can be critical in order to achieve the desired selectivity and sensitivity. This effect is typical for electrospray interfaces. In the antibiotic assays, the extraction potential was maintained between 15 and 17 V. Examples of the chromatograms obtained for cloroxacillin and penicillin G contained in muscle tissue and obtained by single ion monitoring at different m/z values are shown in **Figure 4**. It shows that monitoring at m/z values of 293, 390 and 434 selects the cloxacillin from the accompanying unresolved substances almost exclusively. It would also appear that the best signal-to-noise ratio was achieved employing m/z values of 293 and 434. The apparent magnitude of the signal-to-noise ratio indicates that the assay could detect levels of cloxacillin as low as 40 ng g^{-1}. The optimum extraction voltage for assaying penicillin G in milk appears to be far more critical, the best selectivity being obtained at a m/z value of 333. The lower limit of detection, however, is much smaller and it would appear that an antibiotic level of 1 ng g^{-1} might be detectable.

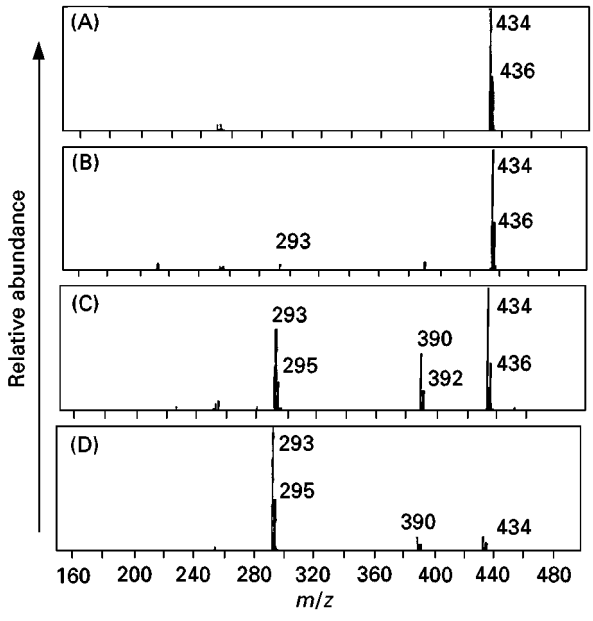


Figure 3 The effect of changing the extraction voltage on the fragmentation pattern. Extraction potential 5 V(A), 10 V (B), 15 V (C) and 20 V (D). (Reproduced with permission from Blanchflower *et al.*, 1994.)

Although the technique of LC/MS is ideal for the detection and identification of drugs and their metabolites in samples of biological origin, it is often

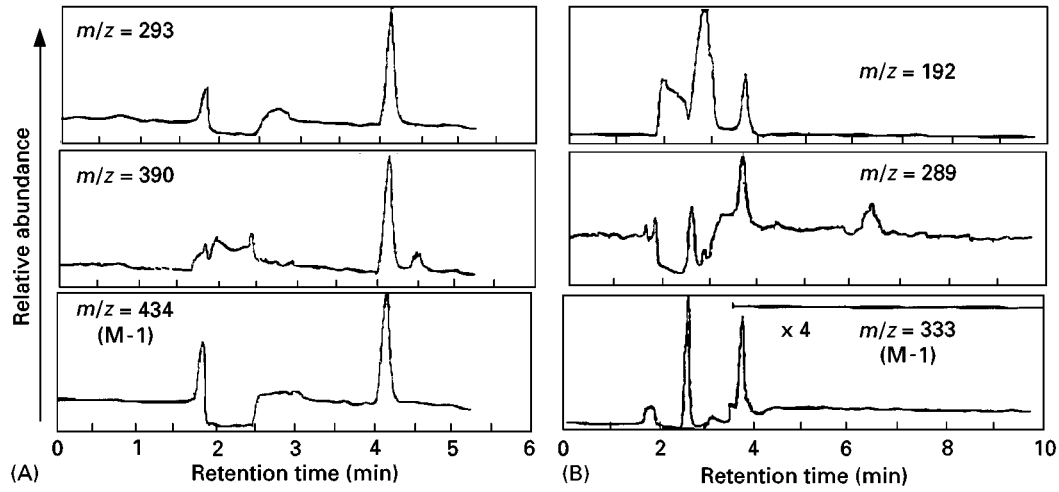


Figure 4 Chromatogram from the assay of cloxacillin and penicillin G monitored at different *m/z* values. (A) Muscle spiked with 200 ng g^{-1} cloxacillin, (B) milk spiked with 4 ng g^{-1} penicillin G. (Reproduced with permission from Blanchflower *et al.*, 1994.)

necessary to use quite complicated sampling procedures. For example, Cai and Henion developed an intricate combination of sampling techniques to determine LSD and its analogues in urine. The urine sample was first subjected to affinity chromatography, which selectively removed the LSD and its metabolites from the urine. The materials isolated on the affinity column were then displaced and collected in a special trap, from which the materials of interest were then again displaced onto the LC column for separation. The column eluent was then passed through an atmospheric pressure chemical ionization interface to the mass spectrometer. A diagram of their apparatus is shown in **Figure 5**. The analytical procedure was quite complicated. The immuno-affinity column that was employed was an HiPac protein G column (3.3 mm × 2.1 mm) packed with 30-μm particles. Prior to use, the column was equilibrated

DropBooks

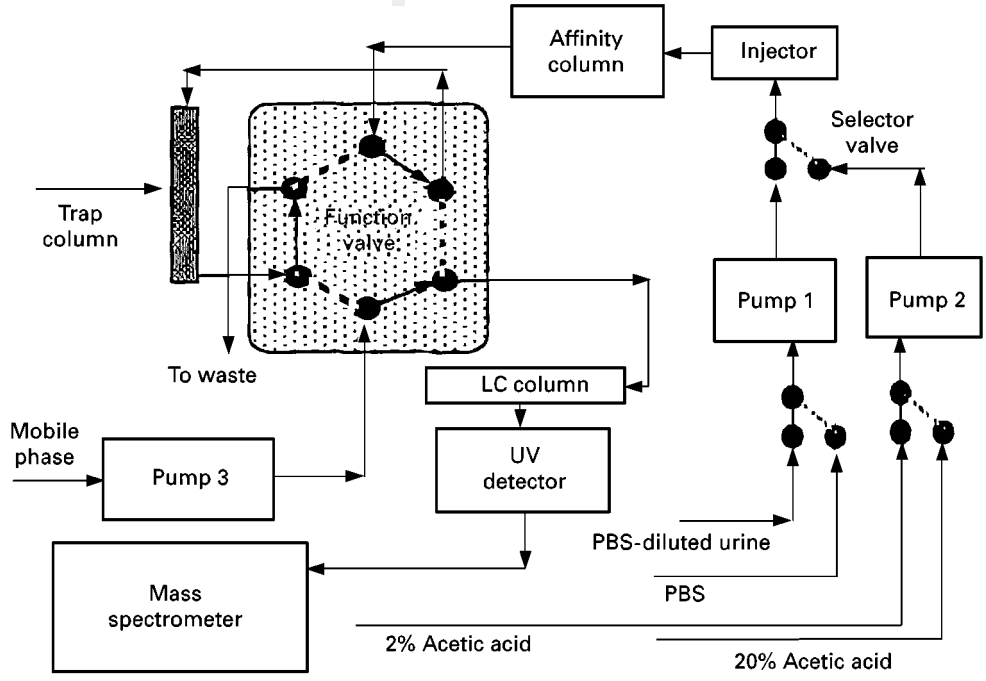


Figure 5 Diagram of the sampling arrangement for the analysis of LSD in urine by an LC/MS. (Reproduced with permission from Cai and Henion, 1996.)

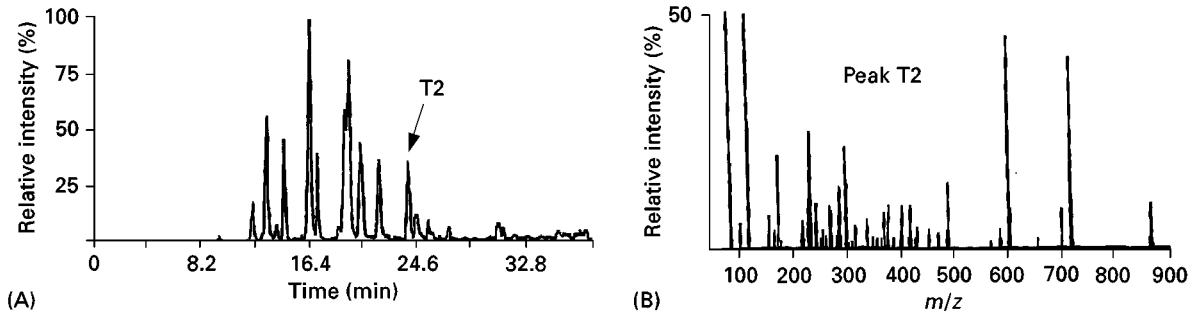


Figure 6 (A) Total ion current chromatogram of a tryptic digest sample of human growth hormone. (B) Spectrum of a product from the tryptic digest of human growth hormone obtained from low dead volume atmospheric ionization interface.

with phosphate buffered saline (PBS). 30 μL of PBS-diluted antibody solution (10% antibody/90% PBS) was then injected onto the column. Then, the sample of human urine, diluted with PBS (50% urine/50% PBS), was pumped through the protein G column and immediately flushed with PBS to remove the weakly bound impurities. While this process was taking place, the trapping column (1.5 cm long, 1 mm i.d.; packed with 5-μm C_{18} particles) and the LC column (15 cm long, 0.3 mm i.d.; packed with 3-μm C_{18} particles) were equilibrated with the mobile phase. The PBS was then pumped through the affinity column and the trap, desorbing the materials from the affinity column and re-adsorbing them on the trap. The trap was then back-flushed and the desorbed materials eluted through the LC column into an API interface and thence into the mass spectrometer. Four metabolic products in addition to the unchanged LSD itself were separated and identified from their mass spectra. The concentration of LSD in the original urine sample was 0.9 ng mL^{-1}. The results of Cai and Henion demonstrate how, by utilizing the selective extraction that is provided by affinity chromatography, very high sensitivities can be obtained.

Recently Thomson *et al.* described a modification of the atmospheric pressure chemical ionization technique involving a special low dead volume interface. Thomson *et al.* employed packed microbore columns (170, 320 and 500 μm i.d., with lengths ranging from 5 to 15 cm) in conjunction with a low-volume, wall-coated capillary column as an interface. The total ion current chromatogram of the tryptic digest sample from about 1 pmol of human growth hormone is shown in **Figure 6**A. The column was packed with an octadecyl bonded-phase having a mean pore size of 300 Å and a particle diameter of 7 μm. The separation was developed by employing a gradient of from 20% solvent (0.1% TFA in water, Figure 6A) to 80% solvent (75% of 0.1% TFA and 25% acetonitrile, Figure 6B) over a period of 1 h. Flow rates of about 80 to 100 μL min^{-1} were used, and about 3 μL min^{-1} of the flow was split from the mainstream and passed to the capillary column *via* the capillary interface. It can be seen from Figure 6 that an excellent separation is obtained and apparently little resolution is lost in the capillary interface. The mass spectrum of the peak marked T2 in the chromatogram is shown in Figure 6B. It is clear that good quality spectra can be obtained for ion masses of up to least 900. Such a combination of techniques can be invaluable for the structural elucidation of compounds generated in biochemical research.

See also: **II/Chromatography: Liquid:** Detectors: Mass Spectrometry. **Extraction:** Solid-Phase Extraction.

Further Reading

Blanchflower WJ, Hewitt SA and Kennedy DG (1994) Confirmatory assay for the simultaneous detection of five penicillins in muscle, kidney and milk using liquid chromatography–electrospray mass spectrometry. *Analyst* 119: 2595–2601.

Cai J and Henion J (1996) On-line immunoaffinity extration-coupled column capillary liquid chromatography/tandem mass spectrometry: trace analysis of LSD analogs and metabolites in human urine. *Analytical Chemistry* 68(1): 72–78.

Cannavan A, Blanchflower WJ and Kennedy DG (1995) Determination of levamisole in animal tissues using liquid chromatography–thermospray mass spectrometry. *Analyst* 120: 331–333.

McCracken RJ, Blanchflower WJ, Rowen C, McCoy MA and Kennedy DG (1995) The determination of furazolidone in porcine tissue using thermospray liquid chromatography–mass spectrometry and study of the pharmacokinetics and stability of its residues. *Analyst* 120: 2347–2351.

Thomson B, Covey T, Shushanm B, Allen M and Sakuma T (private communication) A low dead volume atmospheric ionization interface, Perkin Elmer Corporation.